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Drosophila parthenogenesis: a model for de novo centrosome assembly

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Abstract

The *Drosophila* egg contains all the components required to properly execute the early mitotic divisions but is unable to assemble a functional centrosome without a sperm-provided basal body. We show that 65% of unfertilized eggs obtained from a laboratory strain of *Drosophila mercatorum* can spontaneously assemble a number of cytoplasmic asters after activation, most of them duplicating in a cell cycle-dependent manner. Such asters are formed by a polarized array of microtubules that have their Asp-associated minus-ends converging at a main focus, where centrioles and typical centrosomal antigens are found. Aster assembly is spatially restricted to the anterior region of the oocyte. When fertilized, the parthenogenetic egg forms the poles of the gonomeric spindle by using the sperm-provided basal body, despite the presence within the same cytoplasm of maternal centrosomes. Thirty-five percent of parthenogenetic eggs and all unfertilized and fertilized eggs from the sibling bisexually reproducing *D. mercatorum* strain do not contain cytoplasmic asters. Thus, the *Drosophila* eggs have the potential for de novo formation of functional centrosomes independent of preexisting centrioles, but some control mechanisms preventing their spontaneous assembly must exist. We speculate that the release of the block preventing centrosome self-assembly could be a landmark for ensuring parthenogenetic reproduction.

Keywords: *Drosophila*; Parthenogenetic development; Centrosome inheritance; de novo centriole assembly; Spindle pole formation

Introduction

The centrosome is the main organizing center (MTOC) of animal cells. This organelle nucleates the microtubules that organize the cytoplasmic network at interphase and the bipolar spindle during mitosis. Hence, the centrosome function is basic to ensure the correct position and distribution of many cellular organelles and the proper alignment and successful segregation of chromosomes (Rieder et al., 2001). There is also growing evidence for the involvement of the centrosome in control of cytokinesis and cell-cycle progression (Doxsey, 2001). The animal centrosome is typically composed of a pair of cylindrical structures, called centrioles, that act as target for the recruitment of the pericentriolar material. The centrosome ability to nucleate microtubules resides in the pericentriolar material, by using templates that contain γ-tubulin, a member of the tubulin family (Oakley and Oakley, 1989). The dispersion of the pericentriolar material after centriole disassembly by antibodies to polyglutamylated tubulin suggests that the structural organization of the centrosome is strictly dependent on the centrioles (Bobinnec et al., 1998). Centrosome reproduction is dependent on centriole replication that in higher animals seems to occur only in association with preexisting centrioles (Marshall, 2001). Thus, since the majority of sexually reproducing organisms lose the maternal centrioles during gametogenesis (Schatten, 1994) or as meiosis resumes (Palazzo et al., 1992), the unfertilized oocyte is usually unable to assemble a functional centrosome and cannot develop. The zygotic centrosome, which drives the formation of the first bipolar spindle and the mixing of parental complements, must be of biparental origin. The egg cytoplasm provides the molecular components of the pericentriolar material, while the sperm cells supply the first centriole, around which the pericentriolar material is recruited to organize a functional reproducing centrosome. However, the requirement for the male gamete in early development is not absolute. Many animal eggs can develop to adulthood by parthenogenesis, a special mode of repro-

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duction that occurs without the male contribution. Because of the lack of the supplied sperm basal body, parthenogenetic eggs have to assemble the first centrosome from maternal sources alone. However, despite the potential of this peculiar mode of reproduction for investigating the process of de novo centriole and centrosome formation, the mechanisms involved in centrosome inheritance during parthenogenetic development are still an outstanding question. Two reports have recently exploited this opportunity in haplodiploid hymenopteran eggs showing alternative strategies for zygotic centrosome inheritance (Riparbelli et al., 1998; Tram and Sullivan, 2000). The *Drosophila* egg cytoplasm alone, although it contains all the components required to progress through the early mitotic divisions (Foe et al., 1993) is unable to organize a functional reproducing centrosome, without the sperm-provided basal body, and thus unfertilized eggs never develop beyond meiosis. The *Drosophila* egg can assemble an enigmatic MTOC that seems to be involved in maintaining the orientation of the female meiotic spindle (Riparbelli et al., 2000), like the astral microtubules that orient and position the mitotic spindle within the cell (Wittmann et al., 2001). The female MTOC contains several centrosomal components (Megraw and Kaufman, 2000) but lacks centrioles that were lost early during oogenesis (Mahowald and Strassheim, 1970). So, the female MTOC nucleates an astral array of microtubules, but it cannot reduplicate (Riparbelli and Callaini, 1996; Endow and Komma, 1997). The finding that laboratory strains of *Drosophila mercatorum* can reproduce, although at low rate, by thycoparthenogenesis (Kramer and Templeton, 2001) represents a surprising exception. Studying centrosome inheritance in this system could have important consequences for our understanding of the mechanisms governing the process of centrosome assembly in the absence of preexisting centrioles and could provide additional insights into centrosome inheritance in parthenogenetic insects. Our results show that, after the resumption of meiosis, 65% of unfertilized *D. mercatorum* eggs can spontaneously assemble a number of centrosome-based asters, whereas the remaining 35% of unfertilized eggs and all fertilized and unfertilized eggs obtained from the sibling bisexually reproducing strain never contain cytoplasmic asters. These findings suggest, therefore, that alternate routes for centrosome formation, independent of preexisting centrioles, may be present in the *D. mercatorum* egg.

Materials and methods

Stocks

D. mercatorum stocks used in this work were the parthenogenetic strains *K23-O-im* and *im 43-7* originally isolated by Kramer and Templeton (2001) and the bisexual strain *15082-1521* that was used as control. All the stocks were obtained from the Tucson Stock Center. Since we did not find relevant differences between the parthenogenetic strains, the experiments were referred to the *K23-O-im* stock. The flies were reared on standard *Drosophila* medium at 24°C. Parthenogenetic females from the *K23-O-im* stock were crossed with *15082-1521* control males.

Reagents

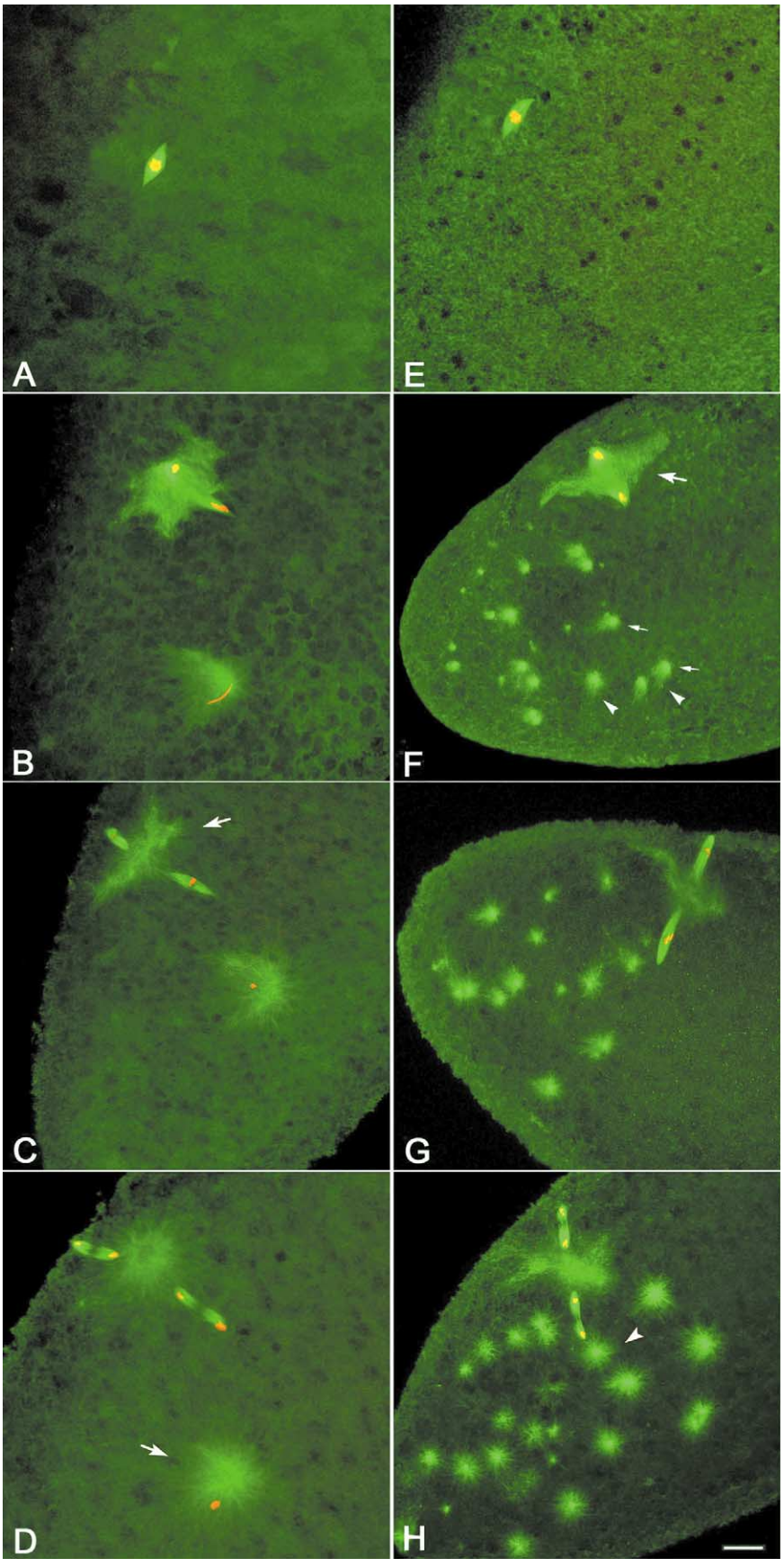
A mouse monoclonal anti-β-tubulin (Boehringer–Mannheim, UK) was used at a 1:200 dilution; a rat monoclonal YL1/2 directed against tyrosinated α-tubulin (Harlan Sera-Lab, England) at a dilution of 1:20; a mouse anti-γ-tubulin monoclonal antibody (Sigma, St. Louis, MO) at 1:100; a rabbit polyclonal anti-Asp serum Rb3133 (Saunders et al., 1997) at 1:50 dilution, a rabbit polyclonal anti-CP190 serum Rb188 (Whitfield et al., 1988) at 1:400 dilution; a rabbit HsCen1p polyclonal antibody (Paoletti et al., 1996) at 1:400 dilution; a rabbit polyclonal anti-centrosomin antibody (Li and Kaufman, 1996) at 1:400 dilution. Goat anti-mouse, anti-rat, or anti-rabbit secondary antibodies coupled to fluorescein or rhodamine (Cappel, West Chester, PA) were used at 1:600 dilution. DNA was stained with propidium iodide, Hoechst 33258 (Sigma, St. Louis, MO) or TOTO-3 iodide (Molecular Probes, Europe, BV). Colchicine, bovine serum albumin (BSA), and ribonuclease A (RNase) were obtained from Sigma.

Drug treatment

For colchicine incubation, just laid eggs were dechorionated in a 50% bleach solution, washed in distilled water, dried on filter paper, and permeabilized with heptane. The eggs were then quickly dried, covered with tissue paper, and incubated with 5 mM colchicine diluted with D20 medium (Echalier and Ohanessian, 1970) for 15 min at 24°C. The tissue paper prevented the embryos from floating on the surface of the solution and ensured that the whole embryo was in contact with the drug. Controls were performed by incubating the eggs without colchicine in D20 medium.

Fluorescence preparations

Metaphase I oocytes and subsequent meiotic and mitotic stages were obtained according to Riparbelli and Callaini (1996) either by dissection of ovaries or collected from 5- to 7-day-old females. Eggs were fixed at 20 min to obtain meiotic stages or held for 30 min or 2 h before fixation to examine later developmental stages. Dechorionated eggs were fixed 10 min in cold methanol, washed in PBS, and incubated for 1 h in PBS containing 0.1% BSA. For double staining of microtubules and asp, CP190, centrosomin, or centrin embryos were incubated overnight at 4°C with the specific antisera and then with anti-β-tubulin antibody for 4–5 h at room temperature. For simultaneous localization of microtubules and γ-tubulin, the embryos were incubated overnight at 4°C with the anti-γ-tubulin antibody, then the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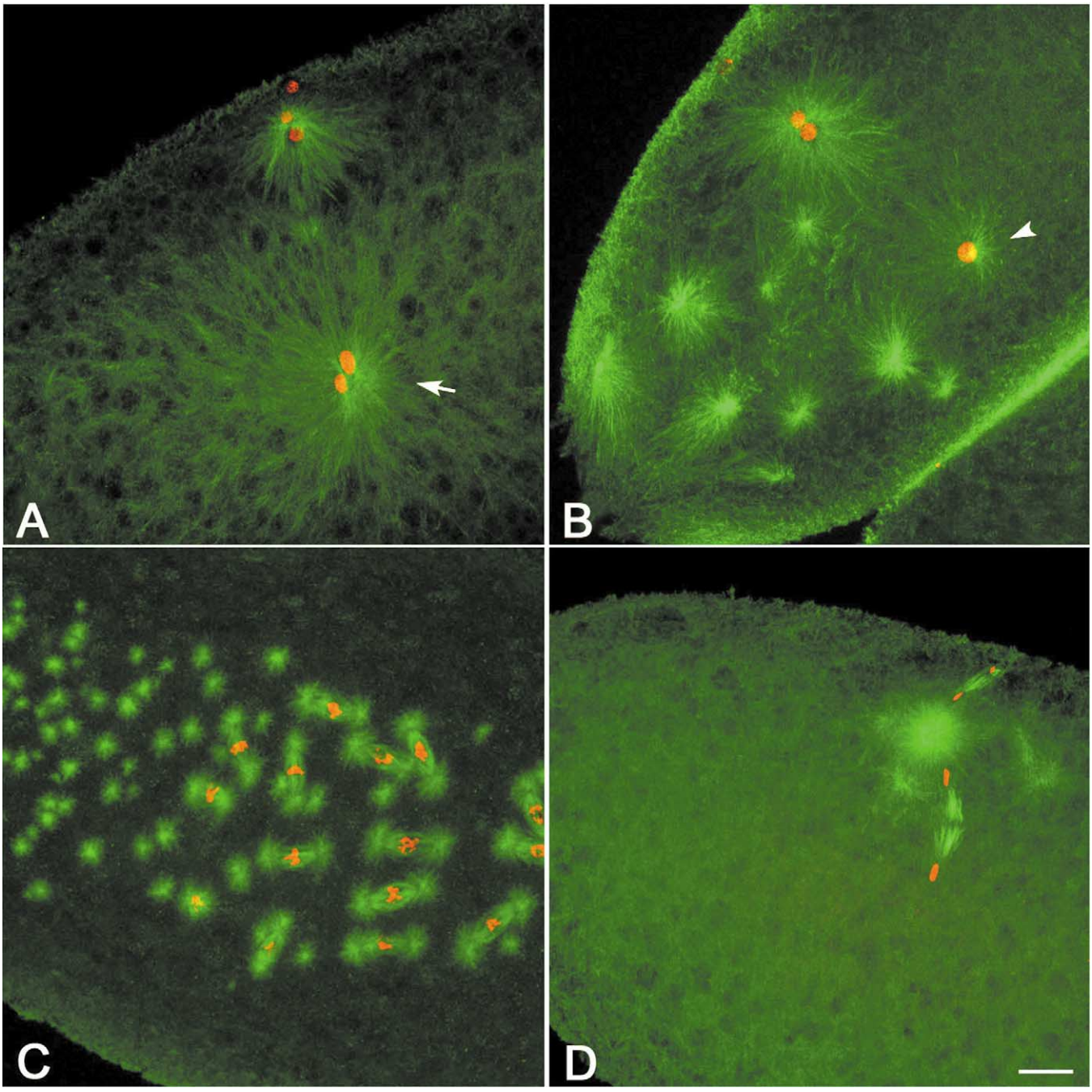


Fig. 2. Completion of meiosis in fertilized (A) and unfertilized (D) bisexual and parthenogenetic (B) eggs and early development (C) in parthenogenetic embryos. Microtubules are revealed by staining with antibodies against β-tubulin (green) and DNA is stained with propidium iodide (red). In fertilized bisexual eggs, the distance between male and female pronuclei decreases and, once they are in contact, a bipolar array of microtubules organizes among them (A, arrow); in unfertilized parthenogenetic eggs, the haploid female pronucleus is associated with one aster (B, arrowhead). (C) Fifth mitosis in unfertilized parthenogenetic eggs: note the anterior clustering of the free cytoplasmic asters. (D) Unfertilized eggs from bisexual females complete meiosis, but lack cytoplasmic asters and do not develop beyond telophase II. Bar, 15 μm.

Fig. 1. Meiosis in fertilized bisexual (left) and unfertilized parthenogenetic (right) eggs. Projected series of optical sections of eggs stained with antibodies against β-tubulin (green) and propidium iodide (red). Metaphase (A, E). Anaphase I (B, F): the meiotic spindle is anastral with long equatorial microtubules (arrow); small arrows and arrowheads point to "head" and "tail" of the comet-like microtubule-based structures observed after oocyte activation. Metaphase II (C, G): twin anastral tandemly aligned meiotic spindles are separated by unfocused spindle pole body microtubules (arrow). Late anaphase II (D, H): the sister chromatids separate and migrate to the opposite poles of the meiotic spindles to form four haploid complements, the more centrally located complement becomes the female pronucleus. A large microtubule aster is associated with the sperm head (arrow) in fertilized eggs, whereas several small asters are observed in unfertilized parthenogenetic eggs; one aster is often found in unfertilized eggs near the more interior female complement starting from anaphase (arrowhead). Bar, 15 μm.

YL1/2 antibody was added and the incubation proceeded for 2 h at room temperature. After washing in PBS–BSA, the embryos were incubated for 1 h with the appropriate secondary antibodies. Controls of the secondary antibodies alone were done for all staining. For simultaneous tubulin and DNA staining, the eggs were incubated for 4–5 h at room temperature in the anti-β-tubulin antibody. After washing in PBS–BSA, the eggs were then incubated in the goat anti-mouse antibody to which 1 mg/ml RNase was added. After washing in PBS, the eggs were incubated 30 min in 1 μg/ml propidium iodide. Eggs were mounted in small drops of 90% glycerol containing 2.5% n-propyl-gallate.

Confocal microscopy

Digital optical sections of whole-mount eggs were examined by using a Leica TCS 4D laser scanning confocal microscope equipped with an argon–krypton Laser and coupled to a Leica DMRBE microscope equipped with 63× PL Apo 1.4 objectives (Leica Lasertechnik, Heidelberg). For double-stained samples, the images of the 2 fluorochrome distributions were recorded separately by averaging 8–16 scans of a single optical section to improve the signal/noise ratio with the pinhole open for maximum resolution using low laser emission to attenuate photobleaching. Images of chromosomes, microtubules, and centrosomes collected at several focal planes were superimposed, merged into a single file, and imported into Adobe Photoshop to adjust size and contrast. Prints were made by using an Epson Stylus Photo color printer.

Results

Meiosis in bisexual and parthenogenetic D. mercatorum oocytes

Meiosis in both bisexual and parthenogenetic *D. mercatorum* oocytes is arrested at metaphase of the first division, and chromosomes are typically positioned at the middle of a tapered spindle lacking astral microtubules (Fig. 1A and E). After the oocyte passes throughout the oviduct, the metaphase arrest is released and meiosis resumes. However, whereas bisexual eggs cannot develop beyond telophase of the second meiosis without male contribution, 8–10% of unfertilized parthenogenetic eggs develop to adulthood.

We cannot examine eggs before 4–5 min after they are laid due to the fixation procedure time; thus, the first meiotic figures we observe are early anaphase I spindles. However, since we see no appreciable difference between the organization of the metaphase and anaphase I meiotic spindles in parthenogenetic and bisexual eggs, the process from metaphase to anaphase I is likely similar in the two egg types. The anastral anaphase I spindle is organized by two discrete sets of microtubules oriented antiparallel to each other and focused at opposite poles. The microtubules are longer than the spindle and bend at its midzone to form an uneven equatorial plate-like structure where they overlap (Fig. 1B and F). The meiosis I spindle elongates and the spindle fibers rapidly reorganize into two tandemly aligned spindles at metaphase of meiosis II. Although the twin spindles are anastral, a large aster of microtubules is observed between the innermost poles (Fig. 1C and G). Whereas in *D. melanogaster* the central array of microtubules is associated with a distinct ring- or disk-shaped structure containing centrosomal proteins, the central aster of *D. mercatorum* is well developed, but does not have a distinct focus for microtubules and the centrosomal proteins are barely detectable (data not shown). By anaphase, the sister chromatids separate and move to the opposite poles of the twin spindles that lose contact with the central aster (Fig. 1D and H). At the beginning of telophase, the spindle microtubules reduce in length and the four haploid sets of maternally derived chromosomes are positioned at the opposite extremities of the spindles. Starting from late telophase II, the meiotic spindles become less evident among the four decondensing haploid female complements, the most centrally located of which will become the female pronucleus (Fig. 2A and B).

Table 1
Frequency of cytoplasmic asters in unfertilized parthenogenetic eggs

	n	m/M	M	SD
Anaphase I	73	1/22	9.1	6.8
Metaphase II	88	1/31	13.9	9.8
Anaphase II	69	1/30	14.2	9.7
Telophase II	117	1/34	12.9	10.6
1° Mitosis	93	2/59	23.8	19.6
2° Mitosis	63	3/71	43.3	22.7

Note. Eggs were fixed in methanol/acetone and stained with antibodies to β-tubulin and Hoechst. *n*, number of eggs scored for each stage; m/M, minimum and maximum number of the asters scored at each stage; M, average number of the asters scored; SD, standard deviation.

A centrosome-mediated microtubule nucleation processes is not expected in unfertilized parthenogenetic eggs, since they did not contain sperm-provided centrosomes. Surprisingly, 65% of the unfertilized parthenogenetic eggs examined after activation display distinct microtubule-based structures, that vary in size and number within the eggs scored. The observation that some oocytes at different stages have only one aster suggests that the process of aster formation could occur at different times during meiosis (Table 1). These structures are never detected in metaphase I-arrested parthenogenetic oocytes ($n = 47$; Fig. 1E), but appear starting from anaphase I. This suggests that the process of aster assembly is closely related to downstream events triggering meiotic resumption. The microtubule-based structures have a comet-like shape during anaphase of the first meiosis with the “head” representing the large focus for the long microtubules of the “tail” and for very short radial microtubules (Fig. 1F). Beginning in meiosis II, symmetric astral arrays of microtubules are instead found (Fig.

1G and H). Optical sectioning reveals that the comet-like microtubule structures are enriched at or near the cortex, while the symmetric astral arrays are mostly located deeper into the egg cytoplasm.

Tubulin staining reveals that the asters assemble spontaneously only in the anterior region of the parthenogenetic egg, whereas the remaining cytoplasm is devoid of these structures (Fig. 1F–H). We found only two exceptional eggs in which the asters form through the whole cytoplasm among all the parthenogenetic eggs scored ($n = 1731$). Which process could lead to the formation of multiple asters in the anterior region of the egg is not clear at present. When present in large numbers, the cytoplasmic asters cluster in the anterior region of the embryo, preventing the nuclei to populate this region during the last intravitelline mitoses (Fig. 2C) and forming embryos with an uneven distribution of the mitotic spindles. These embryos, however, reach later nuclear division cycles, and the asters remain clearly visible in the anterior region of the embryo until 11th and 12th nuclear cycles. The cortical localization of the cytoplasmic asters during the last syncytial mitoses suggest that they can move outwards with migrating nuclei.

To verify whether the ability to assemble cytoplasmic asters is specific to parthenogenetic eggs or is also present in *D. mercatorum* bisexual eggs but silenced by sperm entrance, we fixed unfertilized eggs 20 min after laying and stained them for tubulin. Meiosis resumes in these eggs and the presumptive female pronucleus is selected normally at the end of telophase II (Fig. 2D); however, development does not proceed beyond this stage. Astral arrays of microtubules are never seen in the bisexually unfertilized eggs scored ($n = 189$) (Fig. 2D). So, the spontaneous aster formation is normally prevented in bisexual *D. mercatorum* eggs.

Asters contain true centrosomes that can duplicate and assemble bipolar spindles

To check for the presence of retained maternal centrosomes, we stained mature oocytes arrested at metaphase of the first meiosis with antibodies to proteins commonly found at the *Drosophila* centrosome, such as γ-tubulin, CP190, cnn, HsCen1p, and Asp. With the exception of the Asp antigen that localizes to the microtubule minus ends at both the tapered extremities of the meiosis I spindle, none of the centrosomal proteins were found at the meiotic spindle poles or within the egg cytoplasm in detectable aggregates (data not shown). The same results are obtained with the egg of the bisexual strain. Therefore, the spindle morphogenesis during female meiosis of both parthenogenetic and bisexual eggs of *D. mercatorum* is not dependent on centrosome function, nor are centrosomes observed in the egg cytoplasm during metaphase of the first meiosis; the only organized microtubule structure is the anastral meiotic spindle (Fig. 1A and E). This suggests that the activation process triggers the spontaneous assembly of microtubule-based asters in parthenogenetic eggs, although the metaphase arrested oocytes cannot support a centrosome-mediated microtubule nucleation.

We then wondered whether microtubule-based asters observed in the activated parthenogenetic eggs contain centrosomal proteins. We looked therefore at the distribution of some proteins commonly found at the centrosomes during *Drosophila* mitosis. Immunostaining parthenogenetic eggs for the centrosomal protein CP190 reveals that this protein is localized at the focus for the aster microtubules (Fig. 3A). CP190 is a core component that concentrates at high level to the poles of the mitotic spindles in *Drosophila* cells with some tendency to accumulate around the condensed chromosomes (Whitfield et al., 1988).

Since γ-tubulin is a key component of the microtubule nucleation machinery (Oakley, 2000), we would like to ascertain whether this protein is present at the focus of the cytoplasmic asters. Immunofluorescence analysis revealed that this protein accumulates, indeed, at the focus of the astral microtubules, suggesting a crucial role in the formation of these structures (Fig. 3B).

Because *centrosomin (cnn)*, a core component of the *Drosophila* centrosome, plays a crucial role for the proper organization of γ-tubulin and CP190 at the spindle poles (Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999), we examined the localization of this protein in the parthenogenetic eggs. Several cnn-containing bodies of different size can be seen in the "head" of the comet-like structures observed during anaphase I, whereas small fluorescent spots also colocalize with the tubulin foci within the "tail" (Fig. 3C). Like the other centrosomal antigen, the cnn staining concentrates at the focus of the cytoplasmic asters at the end of meiosis (Fig. 3D) and at the poles of the spindles during mitosis. Conversely, cnn staining is barely detectable or absent in a few of the small asters found during anaphase of the first meiosis (Fig. 3C) and in the smaller asters of the egg interior during later meiotic stages (Fig. 3D), suggesting that these might be organized by a centrosome-independent pathway.

Since the product of the gene Asp has been suggested to contribute to the integrity of MTOCs and to play a role in microtubule bundling at the spindle poles (Avides and Glover, 1999; Wakefield et al., 2001, Riparbelli et al., 2002), we also immunostained parthenogenetic eggs with an anti-asp antibody. We find that Asp is localized to the inside of the microtubule asters, but its signal is more diffuse than that of the antibodies to centrosomal proteins (Fig. 3E). This is consistent with the association of the Asp antigen with the microtubule minus-ends and demonstrates that the cytoplasmic asters are organized by polarized arrays of microtubules with their minus end pointing toward the center and plus ends outwards. This is also supported by the observation that the Asp staining is no longer detected in colchicine-treated embryos (data not shown). The lack of signal following microtubule depolymerization could be due, indeed, to the loss of the Asp pool associated with the minus ends of the centrosome associated microtubules.

To assess whether the asters contain centrin, as generally

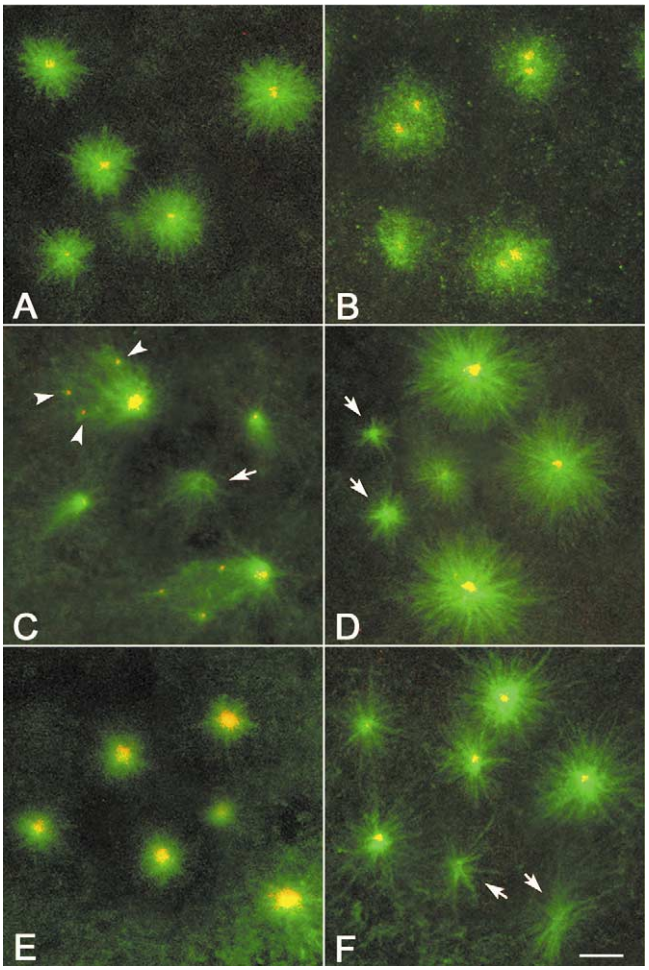


Fig. 3. Centrosomal antigens are localized at the focus of the self-assembled asters. Microtubules are stained green, centrosomal proteins are yellow. Localization of CP190 (A), γ-tubulin (B), cnn (C, D), Asp (E), and centrin (F) within the self-assembled asters during anaphase–telophase of the second meiosis. (C) Detail of the polarized microtubule arrays observed during anaphase of the first meiosis: toward the "head," that contains a large aggregate of cnn, converge longer and shorter microtubules; smaller aggregates of cnn are found within the microtubule bundles of the "tail" (arrowheads). Note that some asters are lacking centrosomal stainings (arrows in C, D, F). Bar, 5 μm.

found at the poles of the mitotic and meiotic *Drosophila* spindles (data not shown), we immunostained laid eggs with an antibody against recombinant HsCen1p human centrin (Paoletti et al., 1996). This antibody recognizes centrin-associated material at the center of the cytoplasmic asters (Fig. 3F). Because the centrin signal suggests that centrioles could be present within the cytoplasmic asters, we examined the ultrastructure of the core region of these asters. Serial sections of 22 cytoplasmic asters from 7 eggs revealed that microtubules converged toward central foci containing electrondense material, and 18 of them also contained centrioles (Fig. 4A).

Because of the finding of the typical centrosomal organization within the cytoplasmic asters, we asked whether the centrosomes are able to duplicate. Labeling with antibodies against β-tubulin during prophase–prometaphase of the first three nuclear division cycles reveals that most of the asters within the embryo cytoplasm are associated in pairs. Moreover, antiparallel microtubules radiating between the opposite asters often partially overlap (Fig. 4B). Thus, a bipolar spindle without chromosomes is formed. The distance between the opposite asters increases at metaphase and anaphase and the bipolar spindle organization is not longer visible (Fig. 4C). During late anaphase, most of the asters display a lager focus. Immunofluorescence analysis revealed that this focus contains a large aggregate of cnn and γ-tubulin, suggesting that centrosome has undergone replication without separation (data not shown). This is consistent with the observation of close twin asters during telophase as if they are nucleated by just separated centrosomes (Fig. 4D). Quantitative analysis suggests coincidence of free aster duplication with mitotic cycles. The average number of the asters doubles, indeed, proceeding from telophase of the second meiosis to first and second mitoses (Table 1).

Sperm centrosome does not prevent spontaneous aster formation, but dictates the organization of the gonomeric spindle

We asked whether the process of aster self-assembly could be affected by the presence of externally provided centrioles by crossing parthenogenetic females with males

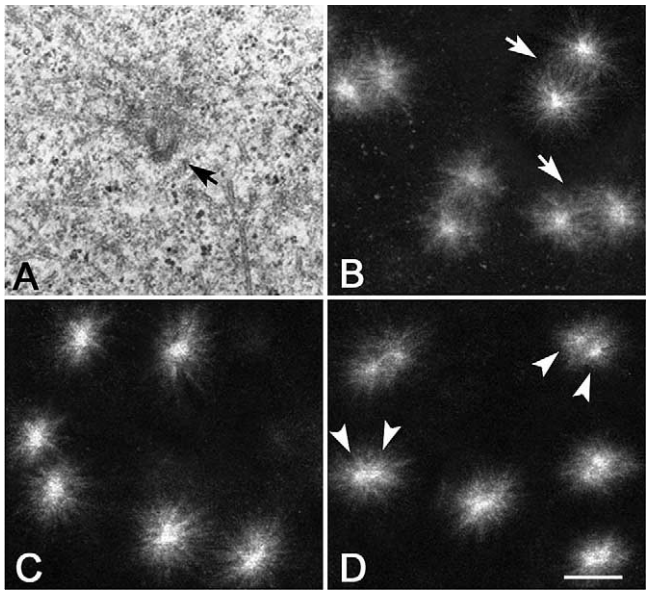
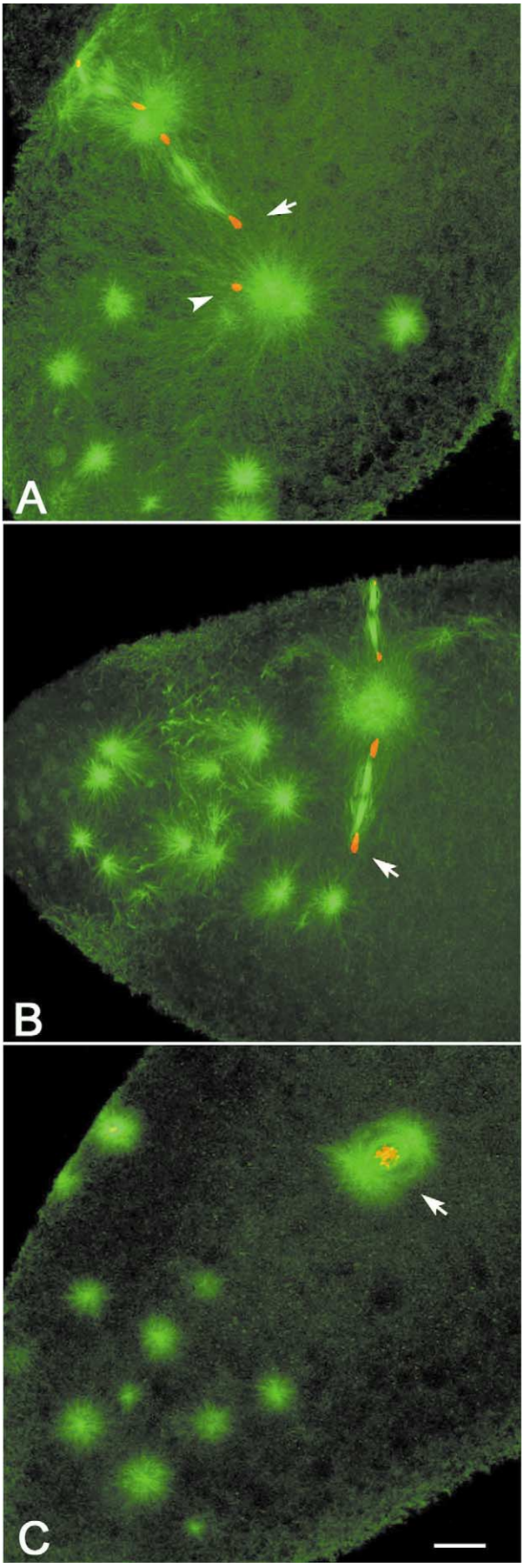


Fig. 4. Asters contain centrioles and can duplicate. (A) Ultrathin section throughout the focus of a cytoplasmic aster showing the presence of centrioles (arrow) and electrondense material, from which microtubules radiate. Microtubule staining of self-assembled asters during the first mitosis: prophase (B), the asters are mostly in pairs and microtubules radiating from opposite poles often overlap to form pseudospindles (arrows); metaphase–anaphase (C): asters among pairs are more distant and the pseudospindles are not longer visible; telophase (D): close pairs of small asters are observed (arrowheads). Bar: 0.15 μm in (A), 4 μm in (B–D).



of the bisexual strain. Although the fertilization rate of this cross is very low (14%; $n = 763$), we observed that the sperm provided basal body does not interfere with aster formation (Fig. 5A). Normally, once in the bisexual egg, the sperm centriole recruits γ-tubulin and other centrosomal proteins and assembles a functional centrosome that is able to nucleate a large astral array of microtubules (Fig. 1B). The sperm aster is irregularly shaped with short microtubules until metaphase II (Fig. 1C). The microtubules then begin to grow, and by anaphase II, they are tightly focused in the proximity of the sperm head (Fig. 1D). The size of the sperm aster increases dramatically at telophase II, and its microtubules reach their maximum length and make contact with the anterior cortex of the egg. This sequential process of sperm centrosome assembly and sperm aster formation is also observed in fertilized parthenogenetic eggs. As telophase II progresses, the sperm centrosome is already duplicated and the sperm aster becomes very large. By contrast, the microtubules associated with the self-assembled asters slightly increase in length both in fertilized (Fig. 5A) and unfertilized parthenogenetic eggs (Fig. 5B). Although self-assembled asters are sometimes observed close to the female pronucleus, only the microtubules associated with the sperm head seem to interact with the putative female pronucleus. Once the parental pronuclei are in contact, their chromatin condenses and a bipolar array of microtubules organizes from the duplicated paternal centrosome. The maternal and paternal chromosomes then congress as individual groups at the equatorial region of the gonomeric spindle (Fig. 5C).

Centrosome-independent spindle formation in unfertilized parthenogenetic eggs

To address how the first mitotic spindle is formed in unfertilized centrosome-free parthenogenetic eggs, we performed a series of observations with different antibodies to microtubules and centrosomal proteins on eggs fixed 30–40 min after laying. At this time, the majority of the bisexual fertilized eggs (85.2%; $n = 334/392$) had completed meiosis and progressed throughout the first mitotic cycle (Fig. 6A–D); 4.1% ($n = 16/392$) of them are completing the second meiosis, whereas 10.7% ($n = 42/392$) had progressed beyond the first mitotic nuclear division cycle.

Fig. 5. The sperm-derived centrosome is dominant over spindle formation in fertilized parthenogenetic eggs, but does not prevent aster self-assembly. Projected series of optical sections of eggs stained with antibodies against β-tubulin (green) and propidium iodide (red). Telophase of the second meiosis in fertilized (A) and unfertilized (B) parthenogenetic eggs: the microtubules associated with the duplicated sperm aster are longer than those of the self-assembled asters; arrow and arrowhead point to the putative female and male pronuclei, respectively. (C) During metaphase of the first mitosis, the parental complements congress separately at the midzone of the biastral gonomeric spindle (arrow) in fertilized parthenogenetic eggs. Bar, 15 μm.

Meiosis resumes in unfertilized parthenogenetic eggs fixed 30–40 min after laying and the female pronucleus is specified, but development progress only in the eggs that display cytoplasmic asters. A total of 81.7% ($n = 330/404$) of these eggs display one mitotic spindle 30–40 min after laying, 4.4% ($n = 18/404$) are completing meiosis, and 13.9% ($n = 56/404$) have progressed beyond the first nuclear cycle. A small aster is frequently observed near the more internal haploid complement starting from metaphase/anaphase of the second meiosis (Fig. 1G and H). A single centrosome is tightly associated with the female pronucleus in parthenogenetic eggs (Fig. 2B) at the time that parental complements in fertilized bisexual eggs meet together and the duplicated sperm-provided centrosome starts to organize the first mitotic spindle (Fig. 2A). This suggests that only monopolar spindles could form in the early zygote. Surprisingly, however, we found that the parthenogenetic eggs scored from metaphase to telophase of the first mitosis assembled bipolar spindles, that fall in two phenotypic classes: monastral (62%; $n = 147/237$) and biastral (38%; $n = 90/237$) spindles.

The formation of the bipolar biastral spindles is consistent with a process of nucleation of antiparallel microtubules from two opposite centrosomes that had migrated along the nuclear envelope (Figs. 6E–H, and 7A and C). Whether the presence of a centrosome pair associated with the female pronucleus is due to an earlier duplication process or is the result of the association of two independently assembled asters to the nuclear envelope is unclear. Two separate findings suggest that a process of centrosome duplication could occur in at least some unfertilized eggs. First, when there are two centrosomes associated with the female pronucleus at prophase, the surrounding free cytoplasmic asters are also in pairs (Fig. 6E), consistent with a nearly synchronous centrosome replication process through the anterior egg cytoplasm. In contrast, when single centrosomes are associated with the female chromatin, most of the free cytoplasmic asters are seen as individuals (Fig. 6I). The dual spindle phenotype could be explained by the different behavior of the centrosomes associated with the female pronucleus, either competent or not to duplicate during prophase of the first mitosis. We cannot, however, exclude that the formation of bipolar spindles could be due to the earlier association of two independently assembled asters with the female pronucleus. The observation during metaphase–anaphase of the first mitosis of a low percentage (9.9%; $n = 9/90$) of bipolar spindles with different sized poles could be in agreement with this possibility.

The monastral bipolar spindles are composed of two half spindles in which only one appears to be organized by a centrosome with a focus of cnn (Fig. 7B). The microtubules in the opposing half-spindle converge to form a large pole that lacks centrosomal antigens. This suggests that the anastral half-spindles are not organized by a canonical MTOC. The alignment of the chromosomes at metaphase indicates that these spindles do consist of two discrete sets of microtubules oriented antiparallel to each other (Fig. 6J). This is consistent with the localization of the Asp gene product on both astral and anastral spindle poles (data not shown). The bipolar monastral spindles can support sister chromatid separation, as inferred by the symmetric anaphase-like position of the chromosomes (Fig. 6K). We also found telophase spindles in which a midbody is formed and daughter nuclei were normally segregated (Fig. 6L). However, only one daughter remains associated with the astral array of microtubules, while the opposing nucleus lacks organized microtubules. Examination with antibodies to cnn reveals that the astral pole at telophase is composed of a close pair of widely separated centrosomes (Fig. 7D) as occurs at the poles of the biastral spindles (Fig. 7C).

Discussion

Examining centrosome assembly in vivo requires, in animal cells, a means of completely eliminating preexisting centrosomes. The few data we have on de novo centriole and centrosome formation in animal cells are provided by in vitro systems or artificially activated eggs (Marshall and Rosenbaum, 2000; Lange et al., 2000; Khodjakov et al., 2002). In this work, we have taken advantage of the fact that unfertilized *D. mercatorum* eggs are able to initiate development by self-assembling centrosome-based asters. Therefore, these eggs provide a means of analyzing centrosome assembly and bipolar spindle formation in a naturally centrosome depleted in vivo system.

Does parthenogenetic development require functional centrosomes?

Parthenogenesis is a self-reproducing mechanism that ensures embryo development without the male contribution in many invertebrates. Since the female gamete lost as a rule maternal centrosomes during oogenesis (Schatten, 1994) or as meiosis resumes (Palazzo et al., 1992), an intriguing question is whether the unfertilized eggs can develop without functional centrosomes or if they have to built centrosomes de novo to undergo embryonic mitosis. Recent studies addressing these questions in two related orders of insects have provided conflicting evidences. Artificially activated unfertilized *Sciara* eggs initiate parthenogenetic development in the absence of centrosomes and bipolar anastral spindles that partially support chromosome segregation form (de Saint Phalle and Sullivan, 1998). By contrast, unfertilized *Muscidifurax* and *Nasonia* eggs can spontaneously assemble in a short time hundreds of centrosome-based asters, on which the formation of the first zygotic spindle relies (Riparbelli et al., 1998; Tram and Sullivan, 2000). However, the development of the unfertilized *Sciara* eggs could not be considered a strictly speaking parthenogenesis, since the embryos never develop to adulthood. Nevertheless, unfertilized *Sciara* eggs represent a useful

model system in which to investigate the acentrosomal pathway of spindle organization during mitosis. By contrast, the *D. mercatorum* unfertilized eggs spontaneously assemble several cytoplasmic asters like those seen in the hymenopteran wasps *Muscidifurax* and *Nasonia*. Immunostaining showed that the focus of these asters contains cnn and CP190 proteins, that have been implicated in the assembly and structural organization of the *Drosophila* centrosome (Whitfield et al., 1988; Li and Kaufman, 1996), and γ-tubulin, a key component of the microtubule nucleation machinery (Oakley, 2000). Transmission electron microscopy analysis confirms that distinct centrioles are, indeed, present at the focus of the cytoplasmic asters. Interestingly, activated frog egg does not develop unless centrioles were supplied artificially (Kuntzinger and Bornens, 2000). These observations are consistent with a crucial role of the centrosome during embryonic development. Although artificially activated eggs can proceed in some cases through the first mitotic divisions in the absence of centrosomes, they never can develop to adulthood. Only unfertilized eggs that can assemble de novo centrosomes have the potential to develop without sperm contribution.

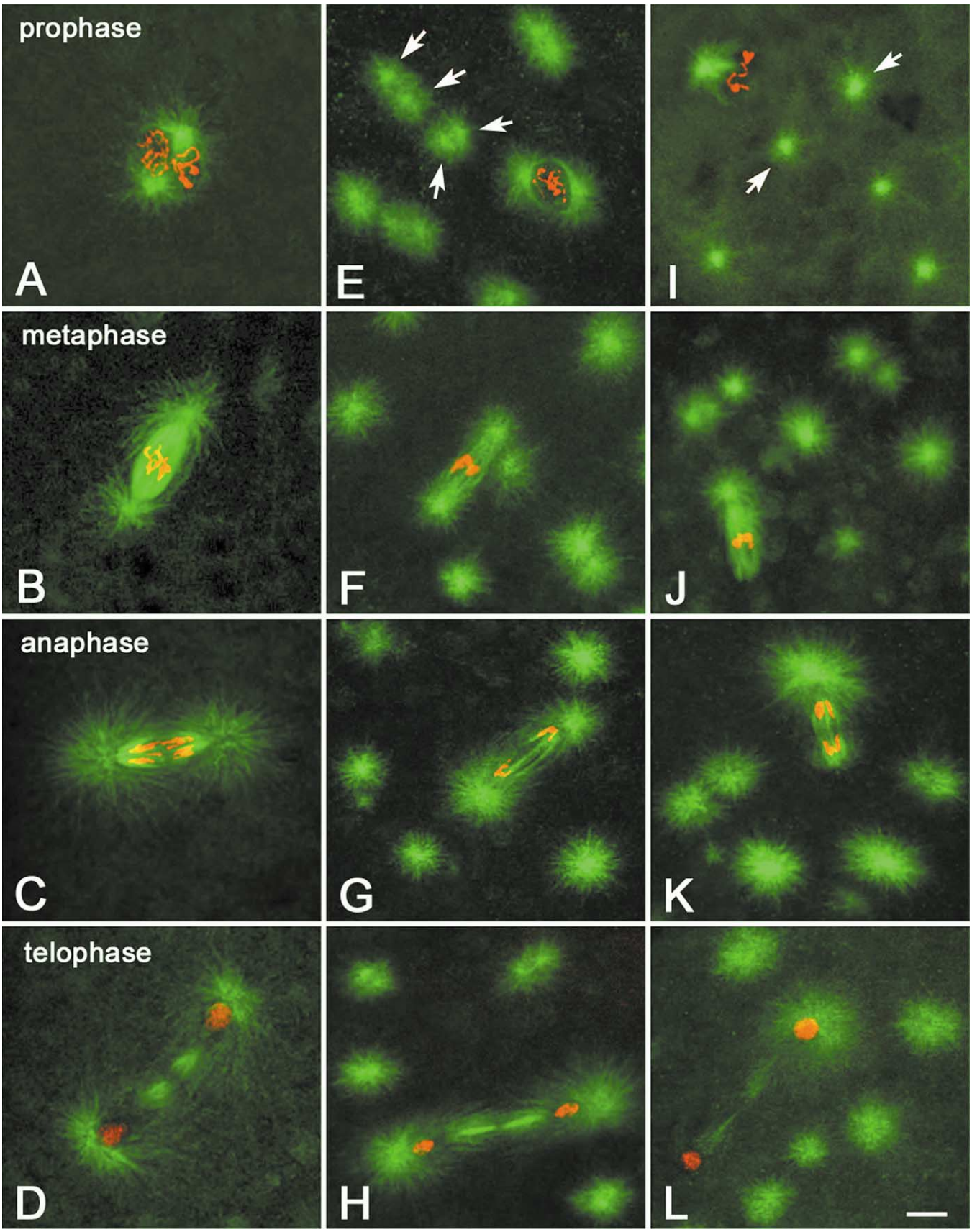
Centrosomal or acetrosomal routes for aster self-assembly?

Motors complexes might sort and focus into discrete asters randomly growing cortical microtubules (Hyman and Karsenti, 1996; Wittmann et al., 2001). Oligomeric motors complexes that can cross-link and move along microtubules have been shown to be sufficient for the self-organization of tubulin into asters in vitro (Nédélec et al., 1997). The motor-based model for aster formation mainly relies on the presence of centrosome-independent nucleated microtubules. Short microtubules are, indeed, enriched in the cortical cytoplasm of the *Drosophila* oocyte, but they are undetected near the center (Therkauf and Hawley, 1992; Page and Orr-Weaver, 1997). The asters are positioned more superficially during anaphase of the first meiosis, the time in which they become evident in the parthenogenetic *Drosophila* egg, than during the following stages. This could allow the self-assembly of small aster at the surface, where a noncentrosomal pathway for their formation is required. These asters can subsequently recruit sparse cytoplasmic components to assemble functional centrosomes that can in turn nucleate long microtubules. According to this model, the centrosomal proteins and centriole precursors would be recruited by the surrounding cytoplasm and carried centripetally along aster microtubules by minus-end motor proteins. This is consistent with previous findings that the accumulation of pericentrin and γ-tubulin at the centrosome in vertebrate cells is inhibited in the absence of microtubules or by microinjection of antibodies to cytoplasmic dynein (Young et al., 2000). The sudden recruitment of γ-tubulin would increase aster dimensions and consequently their ability to attract more centrosomal components. The finding of particles of centrosomal material along the microtubule arrays and the lack of detectable centrosomal antigens at the focus of the smallest subcortical asters at anaphase of the first meiosis supports the recruitment model of centrosome formation. We propose, therefore, that the spontaneous aster assembly follows two sequential steps: first, the reorganization of randomly nucleated microtubules into small asters; and second, the sudden recruitment of centrosomal proteins and the nucleation of larger microtubules. The high concentration of these components at the focus of the asters might represent a suitable environment in which centrioles can self-assemble. Some observations suggest, indeed, that centriole formation might be a later event in the process of the aster centrosome assembly. Centrin is not always present in all asters of the same egg, suggesting that in some cases centrioles are absent or not fully assembled. Additionally, since centrioles are required to concentrate centrosomal material (Bobinnec et al., 1998), the loose pattern of centrosomal antigens found at earlier meiotic phases within the spontaneously assembled asters point to the absence of these organelles. Finally, transmission electron microscopy analysis failed to reveal distinct centriolar structures in 18% ($n = 22$) of the cytoplasmic asters serially sectioned, although electrondense material was present.

Both centrosomal and acentrosomal pathways for spindle assembly are present in the Drosophila egg

Despite the critically important role of the centrosome for proper development, some functions traditionally ascribed to this organelle can be achieved in its absence. Acentrosomal bipolar spindles can form during meiosis of mouse (Calarco-Gillam et al., 1983) and *Drosophila* (Matthies et al., 1996; Riparbelli and Callaini, 1996) oocytes,

Fig. 6. Organization of the first mitotic spindle in unfertilized parthenogenetic eggs. Microtubules are stained green and DNA is red. Progression throughout the first mitosis in fertilized bisexual eggs (left panel) and in unfertilized parthenogenetic eggs (middle and right). Unfertilized parthenogenetic eggs assemble bipolar spindles that can be either biastral (middle) or monastral (right). Prophase: a bipolar array of microtubules organizes among the separated male and female complements in fertilized bisexual eggs (A); in unfertilized parthenogenetic eggs, a bipolar (E) or a monopolar (I) microtubule array is associated with the condensing haploid female chromatin. When two centrosomes are associated with the female pronucleus, the surrounding self-assembled asters are in pairs (E, arrows); by contrast, if only one centrosome is associated with the female chromatin, individuals asters are seen in the cytoplasm (I, arrows). Metaphase: biastral spindles assemble in both fertilized bisexual (B) and unfertilized parthenogenetic (E) eggs; however, the gonomeric spindle holds the male and female chromosomes in bisexual eggs, whereas there is only the haploid female complement at the spindle midzone in parthenogenetic eggs. Chromosome congression at the metaphase plate is also supported by bipolar monoastral spindles (J). Anaphase: sister chromatids move to the opposite poles whether the spindles are biastral (C, G) or monastral (K). Telophase: daughter nuclei form in both biastral (D, H) and monastral (L) spindles. Bar, 4 μm.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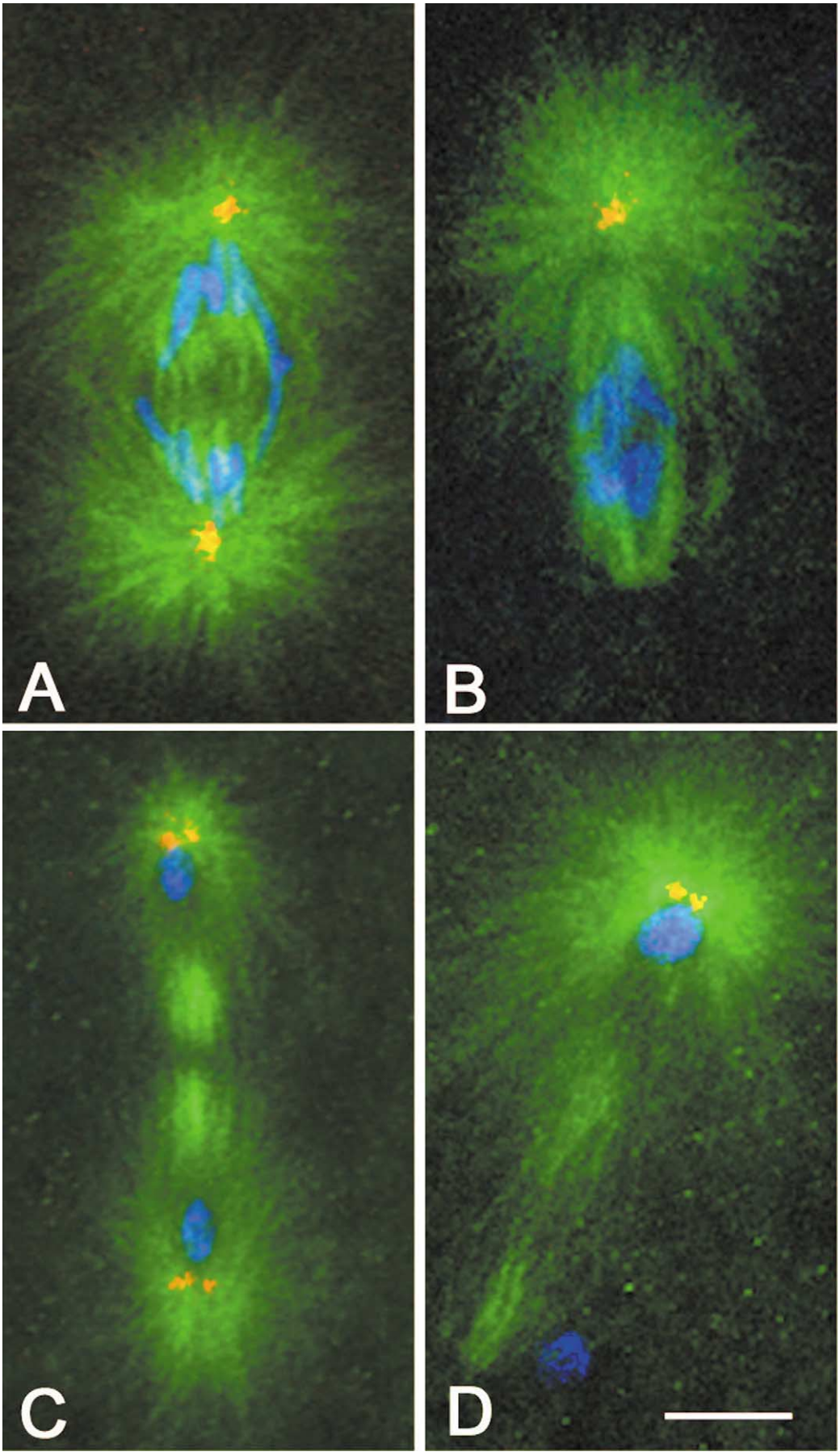
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presumably by result of self-organization properties of microtubules and motors. The ability to assemble spindles in the absence of centrosomes has also been reported recently in neuroblasts from the *Drosophila* mutant *asterless* (*asl*) (Bonaccorsi et al., 2000) and a monkey fibroblast cell line (Khodjakov et al., 2000). Moreover, although mitotic centrosomes do appear to be essential for early *Drosophila* embryonic development (Megraw et al., 1999), null mutants for *centrosomin (cnn)*, which fail to assemble functional centrosomes, can develop into adult flies (Megraw et al., 2001). An intriguing question is, therefore, whether the acentrosomal route of spindle formation is working only when centrosomes are lacking, or is always present but masked by the proper centrosome function. The finding of both normal looking bipolar and anastral bipolar spindles during the intravitelline mitoses of polo[1] *Drosophila* embryo mutants (Riparbelli et al., 2000) points to the possibility that a common cytoplasm can support simultaneously acentrosomal and centrosomal pathways for spindle assembly. This possibility is strengthened by the discovery of monastral bipolar spindles in the parthenogenetic eggs of *D. mercatorum*.

A monopolar spindle would be expected when only a single centrosome associates with chromatin at prophase. Replicated centrosomes that fail to separate properly in certain *Drosophila* mutants function, indeed, as a single unit for microtubule nucleation and assemble monopolar spindles (Rothwell and Sullivan, 2000). A monopolar orientation of the spindle microtubules is, in fact, found when only a single centrosome is associated with the female pronucleus in *Drosophila* parthenogenetic eggs. However, this configuration could be transiently expressed, since we never find monopolar spindles associated with condensed chromosomes during the further phases of the first nuclear cycle. Rather, we observed bipolar spindles in which one pole is astral and contains γ-tubulin, centrin, CP190 and cnn, whereas the opposite extremity is anastral and lacks centrosomal components. Both extremities are stained by the Asp antibody, indicating that microtubule minus-ends converge to the extremities of these spindles, whether or not they are organized by a centrosome. These observations are consistent with recent experiments showing that living vertebrate cells can assemble functional bipolar spindles containing one centrosomal and one acentrosomal pole, after the selective destruction of a centrosome at prophase by laser ablation (Khodjakov et al., 2000). Monastral bipolar spindles were also observed in larval brains of KLP61F *Drosophila* mutants (Wilson et al., 1997). The astral half spindle is organized in *D. mercatorum* eggs by a true centrosome that nucleates astral and kinetochore microtubules, whereas the anastral one could be assembled by chromatin bound microtubules that are focused at their minus ends by motor proteins. This double mechanism of spindle assembly is unexpected. It has been reported, indeed, that the centrosome dictates the preferential route of spindle assembly when there are the conditions to form spindles both with and without centrosomes in *Xenopus* egg extracts (Heald et al., 1997). It could be that centrosome dominance is overcome in the presence of high rate of free microtubule nucleation (Hyman and Karsenti, 1996). However, anastral spindles were formed during the intravitelline mitoses of certain *Drosophila* mutants only in association with chromatin. This suggests that the DNA environment could supply the suitable conditions for local noncentrosomal microtubule assembly. It has been reported, indeed, that the association of the Ran guanosine triphosphate (Ran-GTP) with chromatin induces the self-assembly of spindle microtubules in the absence of centrosomes (Carazo-Salas et al., 1999). Thus, centrosomal and acentrosomal pathways could be simultaneously employed to assemble two discrete sets of microtubules oriented antiparallel to each other.

Parthenogenetic development: an exception to the template model of centriole assembly?

Although the mode of centriole assembly is still a debated and unresolved question, it is clear from our results that in the parthenogenetic egg of *D. mercatorum* the centrioles have to assemble independently of preexisting ones. This is consistent with similar observations in artificially activated sea urchin (Dirksen, 1961; Kallenbach, 1985; Kuriyama and Borisy, 1983; Miki-Noumura, 1977), *Spisula* (Palazzo et al., 1992), parthenogenetic hymenopteran (Riparbelli et al., 1998) oocytes, and vertebrate cultured cells (Khodjakov et al., 2002), where preexisting centrioles are not strictly needed to make new centrioles. How can these data be reconciled with the generally accepted template model, in which the assembly of new centrioles requires the presence of preexisting ones? Recent observations suggest that *Chlamydomonas* cells can assemble centrioles by two pathways: a template pathway and a de novo assembly. The fidelity of centriole duplication during the cell cycle is maintained in this system by a regulatory mechanism that turns off centriole de novo formation in the presence of preexisting ones (Marshall et al., 2001). The assembly of aster-containing centrioles in the presence of sperm provided basal bodies indicates that, in the parthenogenetic *Drosophila* egg, two pathways for centriole assembly are also present, but that they are not mutually exclusive, since sperm do not suppress de novo centriole

Fig. 7. Monastral bipolar spindles are assembled by centrosomal and acentrosomal pathways in unfertilized parthenogenetic eggs. The merged images show DNA (blue), microtubules (green), and cnn (yellow) staining during anaphase (A, B) and telophase (C, D) of the first mitosis. Both the poles of the biastral spindles (left) are organized by distinct centrosomes that duplicate during telophase; by contrast, only one pole of the monastral spindles (right) is organized by a centrosome that duplicates during telophase, whereas the slightly focused opposite pole is anastral and lacks centrosomal antigens. Anaphase progresses and a midbody is formed at the end of telophase in both the spindle types. Bar, 5 μm.

assembly. The maternal centrosomes can also duplicate in a cell cycle-dependent manner in *D. mercatorum* parthenogenetic eggs. Most of them mimic, indeed, the behavior of the spindle-associated centrosomes. Since the process of centrosome duplication and separation mainly relies on centriole replication and splitting, the aster behavior implies the presence of centrioles. The fact that smaller asters often do not show such a duplication process at the same time suggests that they have not yet assembled centrioles at all or that they have only one centriole. During prophase and prometaphase, the sister centrosomes move away and nucleate two astral arrays of antiparallel microtubules that partially overlap. The prometaphase-like spindles, however, do not progress through mitosis, but disassemble in two separated asters as the centrosomes continue to move away during metaphase/anaphase transition. These observations suggest that opposite motor proteins could be associated with the microtubules of the self-assembled asters. This is consistent with computer simulation studies in which motor complexes of opposite directions can lead to antiparallel interaction between overlapping microtubules nucleated by opposite MTOCs (Nédélec, 2002).

Why can *Drosophila* parthenogenetic eggs spontaneously assemble asters, whereas the bisexual unfertilized eggs do not? A trivial explanation would be that control mechanisms ensuring the correct centrosome number are active in bisexual eggs to ensure normal development. Failure to prevent the accumulation of supernumerary centrosomes is, indeed, critical for the egg development, and oocytes have developed several strategies to maintain the proper centrosome number before and at fertilization (Sluder and Hinchcliffe, 1999). The report of spontaneous aster assembly in activated *Drosophila* oocytes, but not in laid eggs (Wilson and Borisy, 1998), is consistent with a working control mechanism, that can be turned off under particular conditions.

The two-step model for centrosome self-formation during *Drosophila* parthenogenesis proposed here mainly relies on the initial formation of astral arrays of microtubules that ensure precursor concentrations high enough to allow de novo centriole assembly and centrosome formation. Any mechanism involved in controlling centrosome-independent microtubule nucleation and aster assembly could be, therefore, a likely candidate for avoiding spontaneous formation of maternal centrosomes and maintaining the correct centrosome number in the egg cytoplasm. Phosphorylation is believed to play a main role in regulating microtubule nucleation and dynamic (McNally, 1999), but it is likely that other mechanisms are also involved in this process. It has been demonstrated, for example, that the Ran-GTP induces the self-organization of microtubule asters in *Xenopus* egg extracts (Ohba et al., 1999). The finding that unfertilized eggs from mutants in Laborc[d], a gene encoding a cytoplasmic heavy chain dynein, spontaneously assemble cortical small asters (Belecz et al., 2001), could point to the involvement of this protein in the mechanisms preventing the formation of multiple centrosomes in *Drosophila*.

Parthenogenetic eggs seem to be able to regulate spatially the aster formation, by allowing aster self-assembly in the anterior region, but not in the posterior region. This raises the question of whether control mechanisms for aster assembly only work in one-half of the embryo, or whether they work on the whole egg but there is an asymmetric distribution of the molecular components required to assemble functional centrosomes during early developmental stages. The assembly of supernumerary sperm asters in the posterior region of the egg during polyspermic insemination (data not shown) and the formation of functional spindles with normal-looking poles during the intravitelline mitoses suggest that the whole egg cytoplasm has the potential to assemble functional centrosomes. The finding of two exceptional eggs in which the asters can assemble throughout the whole cytoplasm, from the thousands examined, may represent the occasional misregulation of the control mechanisms that normally are working in the posterior half of the eggs. Thus, we speculate that the mechanisms monitoring spontaneous aster formation are always active in the posterior half of the *Drosophila* eggs, whereas they could not work properly in the anterior region.

The birth of parthenogenesis: a working model

The successful parthenogenetic reproduction in hymenopteran species, such as *Muscidifurax* (Riparbelli et al., 1998) and *Nasonia* (Tram and Sullivan, 2000), mainly depends on the spontaneous assembly of hundreds of asters from which the first zygotic spindle forms. Therefore, production of microtubule-based asters shortly after activation may represent a selective mechanism for ensuring reproduction without the male contribution. This suggests that *Drosophila* eggs are potentially parthenogenetic and that this special mode of reproduction may arise when the block preventing free asters assembly from the maternal pool has been released. Since parthenogenetic females simultaneously lay two egg types, this reproductive strategy might be lesser refined in *D. mercatorum* or have arisen more recently than in Hymenopterans. The finding of a highly variable number of asters that assemble at different times during meiosis is consistent, indeed, with the presence of a poorly working control mechanism for monitoring an excess of self-assembled maternal centrosomes. Since the formation of the first spindle during parthenogenesis mainly relies on the random association of aster microtubules to the innermost female haploid pronucleus, the higher number of asters seen in Hymenopteran eggs improves the probability that the chromatin will encounter functional centrosomes and assemble the first spindle. In *Drosophila*, instead, very few asters concentrate in the anterior region of the egg where the female pronucleus forms.

The spontaneous assembly of multiple centrosomes raises the question of how the parthenogenetic eggs avoid

consequential defects of development. It has been reported, indeed, that having more than two functional centrosomes results in the assembly of multiple spindles and can lead to abortive development (Brinkley, 2001). Since we rarely observe fusion between neighboring asters, we suspect that they are able to maintain a proper spacing, like astral microtubules, during the intravitelline mitoses (Baker et al., 1993). Presumably, once one aster becomes associated with the female chromatin, it maintains the spacing among adjacent asters, so ensuring proper spindle assembly and behavior. Though in fertilized parthenogenetic eggs centrosomes are available from both the maternal cytoplasm and the sperm, only the paternal ones contribute to the organization of the first mitotic spindle. The mechanism of spatial centrosome exclusion proposed in fertilized *Nasonia* eggs (Tram and Sullivan, 2000), in which the sperm aster approaches to the female pronucleus at the time that the maternal asters are still at the egg cortex, cannot work in *Drosophila* where the centrosomes are all concentrated in the anterior half of the egg. Instead, the sperm centrosome nucleates long astral microtubules that reach the female pronucleus at telophase of the second meiosis, whereas the maternal centrosomes assemble smaller asters. Thus, the larger sperm aster is expected to establish more efficient interactions with the female chromatin. Whether the dominant effect of the paternal-derived centrosome is due in *Drosophila* parthenogenetic eggs to intrinsic factors inherited by the sperm basal body, or it is the consequence of the residual function of a control mechanism, that affects only the microtubule nucleating properties of the maternal centrosomes, it is still unclear. The same mechanisms could block maternal centrosome formation, without affecting paternal centrosome assembly and behavior, during development of bisexual eggs. It has been reported, indeed, that the *Spisula* eggs can distinguish parental centrosomes during the first meiosis, differentially regulating their nucleating properties on the basis of molecular markers that provide paternal and maternal identities (Wu and Palazzo, 1999).

Eggs of obligate sexually reproducing organisms have all the components needed to assemble functional centrosomes (Schatten, 1994). Thus, unfertilized eggs could have the potential to develop parthenogenetically, unless there are control mechanisms preventing the spontaneous assembly of maternal centrosomes. The case of *Drosophila* and Hymenopterans suggests that parthenogenesis might occur when these control points are overcome. The mechanisms preventing maternal centrosome formation are crucial to ensure successful fertilization and genome variability, by suppressing attempts at parthenogenetic development. In *D. mercatorum*, the very low developmental success rate of the parthenogenetic eggs is still enough to establish self-reproducing female strains (Kramer and Templeton, 2001), that have high levels of homozygosity at all loci examined (Carson et al., 1969). Thus, Dipteran and Hymenopteran species have made independent attempts at parthenogenetic development by activating analogous routes for de novo centrosome formation. Thus, insect species belonging to different orders may have the same regulatory mechanisms to control similar developmental pathways. The spontaneous aster assembly in artificially activated sea urchin and *Spisula* oocytes suggests that these control mechanisms may be evolutionarily conserved.

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